TOXICITY, LOCALIZATION AND ELIMINATION OF THE PHOTOTOXIN, ALPHA-TERTHIENYL, IN MOSQUITO LARVAE

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ABSTRACT. Mosquito larvae were examined to determine interspecific and interstrain differences in susceptibility to the larvicidal effects of the plant-derived phototoxin, alpha-terthienyl (α-T). The LC_{50} values were as follows: *Aedes aegypti*, 4 ppb; *Ae. epactius*, 6 ppb; *Anopheles stephensi*, 14 ppb; malathion-susceptible *Culex tarsalis* (S), 12 ppb; malathion-resistant *Cx. tarsalis* (R), 16 ppb. Fluorescence studies indicated localization of α-T in the midgut epithelium and in the lumen of Malpighian tubules. Rates of elimination of tritiated α-T differed significantly between *Ae. aegypti* and *Cx. tarsalis* (S) larvae. Rate of ^{3}H-α-T elimination was inversely correlated with susceptibility to the toxic effects of the compound. The toxicological significance of selective α-T accumulation and the importance of α-T elimination in determining sensitivity are discussed.

INTRODUCTION

Alpha-terthienyl (α-T) is a plant-derived phototoxin which is potentially useful as a mosquito control agent as it is highly toxic to larvae in the presence of sunlight or near ultraviolet light (e.g., LC_{50} = 19 ppb for *Aedes aegypti* (Linn.), Arnason et al. 1981b). Because α-T has a degradation half-life of approximately 4 hours in sunlight (Philogene et al. 1985), it is non-residual and environmentally safe. It is important to note that toxicity has been reported in certain non-target organisms such as tadpoles (Kagan et al. 1984) and fish (Kagan et al. 1986). Arnason et al. (1981a) demonstrated that, upon irradiation of α-T with light of near ultraviolet wavelengths (300–400 nm), photodegradation occurs releasing strongly oxidizing singlet oxygen by a Type II mechanism described by Spikes and Straight (1987). This highly reactive oxygen species is known to have toxic effects on a variety of biomolecules. Singlet oxygen can cause peroxidation of phospholipids and oxidation of enzymes and other proteins (Spikes and Straight 1987). Arnason et al. (1987) observed that exposure of *Aedes atropalpus* (Coq.) larvae to α-T under photosensitizing conditions led to increased halide leakage into the medium and caused visible damage to anal gill membranes. Furthermore, Wat et al. (1980) reported toxic effects of α-T on human erythrocytes, including hemolysis and potassium ion leakage. These authors suggest that membranes may be an important site of α-T toxicity. Although the above findings lend insight into possible mechanisms of toxicity, their importance in determining lethality of the compound has yet to be established.

The fluorescent activity of α-T under near ultraviolet irradiation facilitated the use of fluorescence microscopy to examine sites of α-T localization within mosquito larvae. This report focuses on results of localization studies as well as toxicity tests using four mosquito species: *Aedes aegypti, Ae. epactius* Dyar and Knab, *Anopheles stephensi* Liston, and malathion-resistant (R) and susceptible (S) strains of *Culex tarsalis* Coquillett. With the possible exception of *Ae. epactius*, the species are important vectors of human pathogens (Harwood and James 1979). In addition, the basis for interspecific variation in sensitivity to the phototoxin was examined by comparing rates of elimination of ^{3}H-α-T in *Ae. aegypti* and *Cx. tarsalis* (S).

METHODS AND MATERIALS

Insect rearing. Larvae used were from stock colonies of mosquitoes maintained in the laboratory. Adults were housed in wire screen cages (0.3 m^3) in a 17:7 (L:D) photoperiod. *Aedes aegypti* was kept at 28°C and all other species were maintained at 21°C. Adults were supplied with a 20% sucrose solution and periodic blood meals were provided from restrained guinea pigs. Oviposition took place in 300 ml bowls containing tap water. Eggs were hatched in enamel basins containing dechlorinated tap water and larvae were fed on finely ground TetraMin® fish food (TetraWerke).

Toxicity tests. Groups of 20 third- and fourth-instar larvae were placed in glass shell vials containing 25 ml deionized tap water. Alpha-terthienyl was prepared as described in Philogene et al. (1985). An appropriate amount of stock solution (100 ppm α-T in 95% ethanol) was combined with fresh ethanol in 2 ml beakers to produce 1.0 ml of solvent. Each beaker was poured into a corresponding 300 ml glass bowl containing 225 ml water and was stirred to provide a uniform solution. The glass vials containing 20 larvae were then simultaneously poured into corresponding glass bowls (each bowl thus containing 20 larvae in 225 + 25 ml water + 1 ml α-T solution). The larvae were allowed to

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absorb the toxin for 10 min in darkness. The control group consisted of 20 larvae in 250 ml water and 1 ml ethanol without phototoxin. The larvae were then irradiated for 4 hr using four 20W Westinghouse blacklight blue tubes (F20T12/BLB) with an intensity of 5 W/m^2 in the 300–400 nm range. Larvae were maintained in darkness for a further 20 hr, and the 24-hr mortality was assessed. Dose-response curves and 95% fiducial limits for LD_{50} values were determined using the method described by Matsumura (1975).

Localization studies. Fourth-instar *Culex tarsalis* (S) larvae were immersed in a 10 ppm aqueous α-T solution for 30 min. They were then rinsed and maintained in clean water in darkness. Both whole mount and dissected alimentary tract preparations were used. Micrographs were taken with a Leitz Dialux 20 fluorescence microscope equipped with a 35mm camera, a BP 450-490 exciting filter and an LP 515 suppression filter. The Ilford HP5 panchromatic film was exposed and processed at ASA 1600.

Elimination studies. Fourth-instar larvae were used for determination of clearance profiles using tritiated α-T prepared as described by Iyengar et al. (1987). Specific activity of the tritiated solid was 1.10 mCi/mmol. Stock solution (100 ppm ^{3}H-α-T in 95% ethanol) was added to 250 ml deionized water to give a 100 ppb treatment medium. Approximately 250 larvae were strained in gauze and immersed in the treatment medium for 30 min. Gentle aeration was provided to facilitate uniform mixing. Following the treatment period, larvae were again strained in gauze and rinsed thoroughly with 1 liter deionized water. Larvae were then placed in an enamel basin containing 2 liters deionized water and TetraMin fish food. They were then kept in darkness for the remainder of the study. The water in the basin was changed at 3, 6 and 12 hr posttreatment, and every 12 hr thereafter. At various intervals following treatment, samples of 15 larvae were taken and placed on a 3.2 cm disc of Whatman 3MM filter paper and were "sandwiched" with a second filter paper disc. Samples were squashed and then dried at 60°C for 1 hr. Dried samples were placed in scintillation vials and digested with 0.5 ml of Protosol tissue solubilizer (New England Nuclear Corp.) for 3 hr at 60°C, and 10 ml scintillation cocktail [4 g PPO (2,5-diphenyl-1,3 oxazole), 0.1 g POPOP (1,4 bis-2-5 (5-phenyloxazoyl)-benzene/1 toluene] was then added to each vial. One drop (ca. 20 μl) glacial acetic acid was added to minimize chemiluminescence. Samples were allowed to stabilize in the dark for 3 hr before counting in a Beckman 9000 liquid scintillation counter. Counting efficiency was determined using the external standards method and all data were converted to disintegrations per minute (DPM). Counting efficiency was approximately 45% in this system.

RESULTS AND DISCUSSION

Toxicity tests. Dose-response curves for all species are shown in Fig. 1. The LC_{50} values were as follows: *Ae. aegypti*, 4 ppb; *Ae. epactius*, 6 ppb; *A. stephensi*, 14 ppb; *Cx. tarsalis* (S), 12 ppb; *Cx. tarsalis* (R), 16 ppb. Zero mortality was observed in controls for all species tested.

The difference in α-T sensitivity between the two strains of *Cx. tarsalis* was significant as determined by non-overlap of the 95% fiducial limits ($P = 0.05$). However, it is small in comparison with the difference in malathion sensitivity of these two strains, as their LC_{50} values for malathion differ by two orders of magnitude (Ziegler et al. 1987). This minimal cross resistance of *Cx. tarsalis* (R) to α-T may be due to enhanced activity in this strain of an enzyme which is associated with malathion resistance and is capable of α-T biotransformation as well.

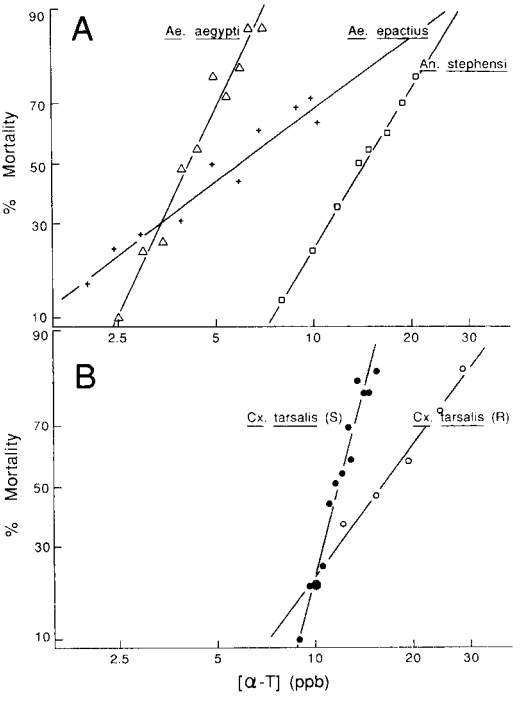

Fig. 1. Twenty-four hour α-T dose-response curves for fourth instar larvae under near-ultraviolet illumination. Fig. 1A: *Aedes aegypti*, Δ; *Ae. epactius*, +; *Anopheles stephensi*, □. Fig. 1B: malathion susceptible *Culex tarsalis* (S), ●; malathion resistant *Cx. tarsalis* (R), O. n = 20. Points represent the average of duplicate trials.

Fluorescence studies. Fluorescence was observed in the midgut and Malpighian tubules of *Cx. tarsalis* (S) larvae (Fig. 2). Fluorescence in the midgut indicated that a portion of α-T was ingested during filter feeding, although passive absorption through the cuticle could have occurred since α-T is highly lipophilic (McLachlan et al. 1986). Fluorescence in the midgut was localized in the peripheral region of epithelial cells (Fig. 3), suggesting an accumulation of α-T either in the cell membrane or in intercellular spaces. The high lipophilicity of the compound supports the former. Furthermore, the toxic effects of α-T on membranes (see Wat et al. 1980) suggest that accumulation in midgut epithelial membranes may lead to selective toxicity in this tissue.

Close examination of the distal tip of a Malpighian tubule *in vitro* (Fig. 4) indicated a concentration of α-T in luminar spaces external to the large tubule cells. Similarly, an examination of the proximal region of Malpighian tubules also showed accumulation of α-T in the lumen (Fig. 4). These findings suggest that Malpighian tubules were sequestering α-T, or a fluorescent metabolite, from the hemolymph and secreting it into the lumen. As with midgut epithelial cells, evidence of α-T accumulation in Malpighian tubules suggests that larvicidal activity of the compound may be based in part on selective toxicity in the organ.

Elimination studies. Fluorescence studies suggested that active excretion of α-T by the Mal-

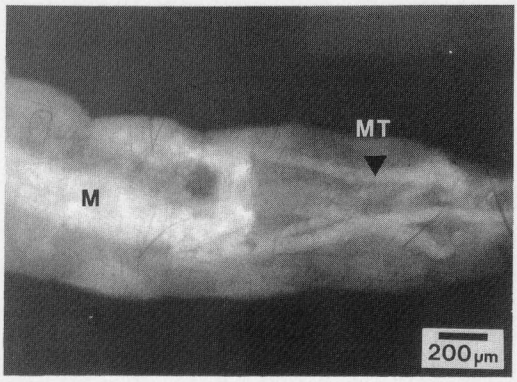

Fig. 2. Fluorescence micrograph of the abdominal region of a 4th instar *Culex tarsalis* (S) larva under ultraviolet illumination 24 hr following α-T treatment. Anterior end is to the left. M, midgut, MT, malpighian tubule.

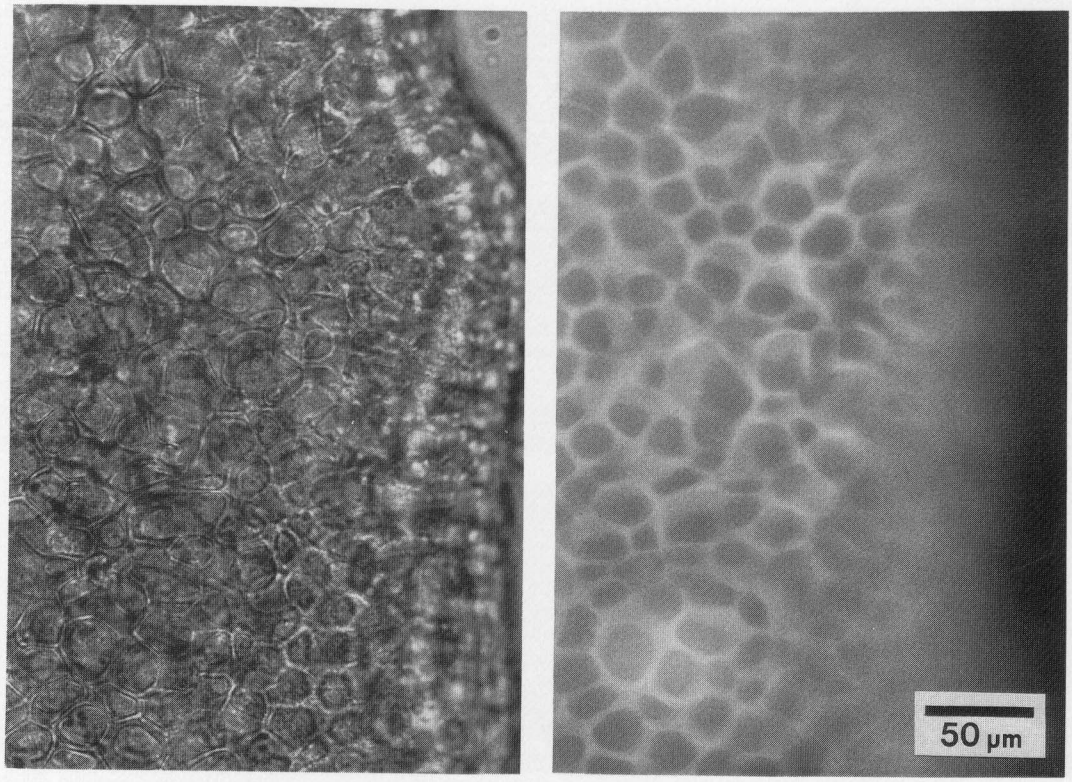

Fig. 3. Basal surface of the posterior midgut wall of a 4th instar *Culex tarsalis* (S) larva 3 hr following α-T treatment. Incandescent + ultraviolet illumination, left; ultraviolet illumination, right.

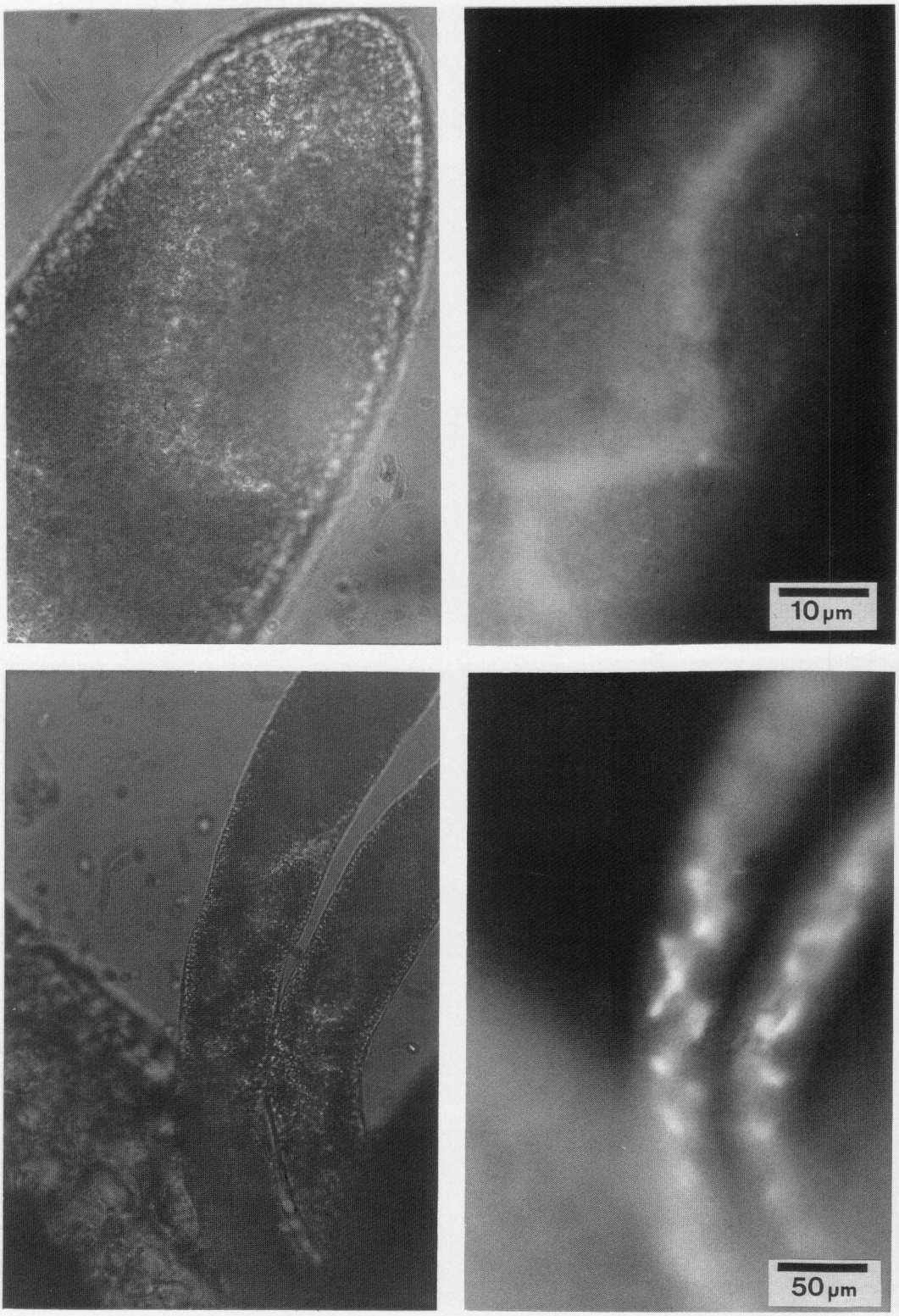

Fig. 4. Malpighian tubules of a 4th instar *Culex tarsalis* (S) larva 2 hr following α-T treatment. Distal region, above; proximal region, below. Incandescent + ultraviolet illumination, left; ultraviolet illumination, right.

pighian tubules occurred. To determine if α-T sensitivity was related to the ability of larvae to eliminate the compound, species with relatively high and low sensitivity to α-T toxicity (*Ae. aegypti* and *Cx. tarsalis* (S), respectively) were used in a comparative ^{3}H-α-T elimination study. The time-dependent clearance profiles for the two species are shown in Fig. 5. These data were analyzed using the polyexponential stripping program, ESTRIP, which calculates goodness of fit for various curves which represent the sum of 1 or more exponential equations, each of which may be expressed as:

$$C = C_0 e^{(-ke \cdot t)}$$

where C is the internal level of a compound at time t, C_0 is the initial level of compound (at time zero) and k_e is the rate constant of elimination (Brown and Manno 1978). In this case, curves representing the sum of 3 such functions provided the best fit. When expressed on a log scale (Fig. 5), these exponential functions are linear. The negative slope of these linear functions gives the rate constant k_e, corresponding to rate of elimination for each function in the curve. The values given for k_{e_1} were calculated using data from the first hour of the experiment, k_{e_2} represents data from 2 to 12 hr posttreatment, and k_{e_3} represents data taken from 16 to 72 hr posttreatment.

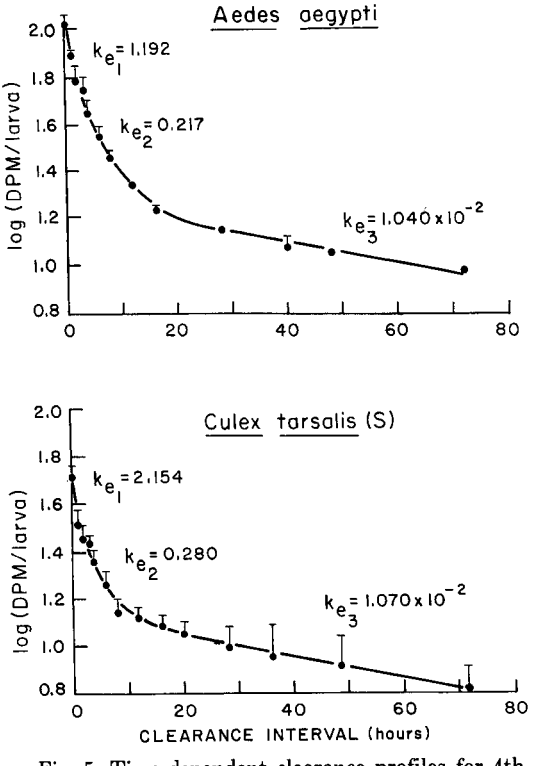

Fig. 5. Time-dependent clearance profiles for 4th instar *Aedes aegypti* (above) and *Culex tarsalis* (S) (below) treated with ^{3}H-α-T. DPM: disintegrations per minute; k_e: rate constant of elimination (see Results and Discussion for explanation). n = 15. Points represent the average of duplicate trials. Vertical bars represent standard error of the mean.

A comparison of initial elimination rates (k_{e_1}) between the two species indicated that, in the first hour, the amount of ^{3}H-α-T eliminated by *Cx. tarsalis* (S) was almost twice that eliminated by *Ae. aegypti* (Fig. 5). A difference in rate constant (k_{e_2}) between species also occurred, but to a lesser extent, between 2 and 12 hr posttreatment. k_{e_3} values indicated no difference in rate constant from 16 to 72 hr. Each rate constant in an elimination profile may represent a functionally discrete compartment (Janku 1971). Compartments may be identified with certain structures (e.g., the midgut of the mosquito larva) but may not necessarily be anatomical in nature. They may represent aqueous or lipid-bound phases of a compound, or different chemical states of a compound, such as the parent molecule and its metabolite (Janku 1971). Regardless of the nature of these compartments, each will have its own characteristic rate of elimination. Since their exact nature in *Ae. aegypti* and *Cx. tarsalis* is not yet clear, a rigorous examination of α-T kinetics in each compartment has not been conducted. Results demonstrate, however, that α-T sensitivity correlates inversely with the elimination rate constant for some of these compartments. Since the difference in rate constant between species was greatest immediately following treatment, α-T sensitivity may be related in part to the inability of the insect to reduce internal levels of the compound before extensive toxification via irradiation occurs. Iyengar et al. (1987) observed that fecal elimination of topically applied α-T was more rapid in less sensitive lepidopteran species such as *Ostrinia nubialis* (Hübner) than in highly sensitive species such as *Manduca sexta* (L.). These findings, as well as results obtained for *Ae. aegypti* and *C. tarsalis* (S), suggest that the ability to rapidly eliminate α-T is a basis for reduced sensitivity to the compound. Enhanced elimination of lipophilic agents such as α-T is most likely a result of increased biotransformation of the compound to a more hydrophilic state.

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